# Membrane targeting: **What a difference a G makes**

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**Pleckstrin homology domains are modular domains that direct membrane targeting of their host proteins by binding to polyphosphoinositides; recent results have increased our appreciation of how some of these domains actually bind 3-phosphoinositides, and along the way thrown up some unexpected observations.**

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The last few years have seen the emergence of 3-phosphoinositides, such as phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$) and phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$), as *bona fide* lipid second messengers [1]. Barely detectable in resting cells, these lipids are produced by phosphoinositide 3-kinases (PI 3-kinases) in response to activation of almost all known cell-surface receptors. One developing theme is that 3-phosphoinositides activate downstream signalling through the rapid recruitment of specific target proteins to the plasma membrane [2].

The small modular pleckstrin homology (PH) domain plays a vital role in membrane targeting of this kind [3]. Of the 100 or more PH domain-containing proteins so far identified, a minority (approximately 10%) bind 3-phosphoinositides with high affinity. Of these, some recognise only $PIP_3$, while others bind equally well to $PIP_3$ and $PI(3,4)P_2$. As agonist-stimulated $PIP_3$ accumulation is immediate and transient, whereas $PI(3,4)P_2$ accumulation is delayed and more sustained [1], differential rates of signalling can be achieved, depending on the specificity of the PH domain present in the host protein. A key issue, therefore, is how PH domains selectively recognise different 3-phosphoinositides. New insights into this issue have come from the recently determined structures of ligand-bound forms of the $PIP_3$-specific PH domains from Bruton's tyrosine kinase (Btk-PH) [4] and the 'general receptor for phosphoinositides-1' (Grp1-PH) [5,6], and of the $PIP_3$/$PI(3,4)P_2$-binding PH domain from the 'dual adaptor for phosphotyrosines and 3-phosphoinositides-1' (DAPP1-PH) [5].

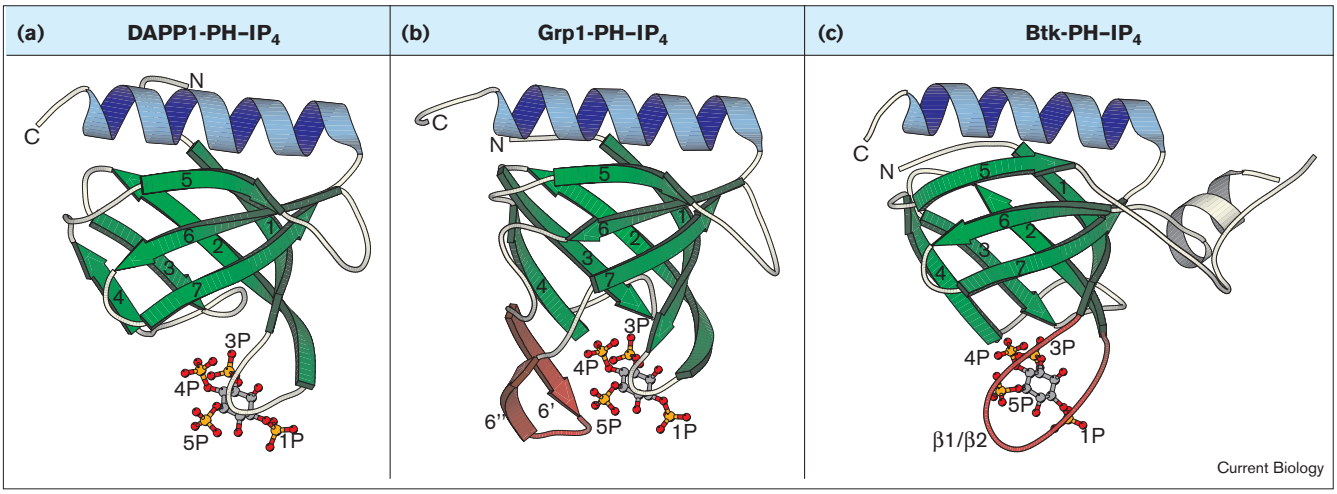
In contrast to other modular signalling domains, the sequence identity between different PH domains is very low, typically 10–20%. In view of this, it is remarkable that the PH domain structures solved to date all have essentially the same fold. The PH domain core has two orthogonal antiparallel β sheets of four (β1–β4) and three (β5–β7) strands, which are closed off at one corner by an amphipathic carboxy-terminal α helix, and at the other corner by the positively charged β1/β2, β3/β4, and β6/β7 loops (see Figure 1). These three loops are the most variable in length and sequence.

As expected, the DAPP1-PH, Btk-PH and Grp1-PH domains share this common fold [4–6]. But whereas DAPP1-PH has no additional elements of secondary structure, Btk-PH and Grp1-PH do have extra elements. Grp1-PH has an insertion in its β6/β7 loop that forms two additional strands (termed β6′ and β6″ in [5]), whereas Btk-PH has no insertion in the β6/β7 loop but instead has an elongated β1/β2 loop. In both cases, these structural variations have the effect of deepening the phosphoinositide-binding pocket. The importance of this becomes apparent when examining the ligand-bound complexes.

In each case [4–6], inositol 1,3,4,5-tetrakisphosphate ($IP_4$) — the water-soluble headgroup of $PIP_3$ — is seen to bind at the corner formed by the β1/β2, β3/β4, and β6/β7 loops. Of the interactions made with $IP_4$, approximately 75% are common to the three different PH domains. These involve only the 1-, 3- and 4-phosphates, but by themselves they appear to be insufficient for high-affinity binding. It is the remaining 25% of interactions, unique to each PH domain, which are required to boost the binding affinity. In DAPP1-PH, the unique interactions are all made with the 4-phosphate, whereas in Grp1-PH and Btk-PH most are made to the 5-phosphate. In effect, the 5-phosphate contributes very little to $IP_4$ binding to DAPP1-PH, consistent with this PH domain binding both $PI(3,4)P_2$ and $PIP_3$.

The manner in which the Grp1-PH and Btk-PH can interact with the 5-phosphate is particularly interesting. In both of the $IP_4$-bound Grp1-PH and Btk-PH complexes [4–6], a clear binding pocket for the 5-phosphate is observed. In Grp1-PH, this pocket is formed primarily from residues of the unique β6/β7 insertion, whereas in Btk-PH, the longer β1/β2 loop is able to envelop the 5-phosphate to make the necessary interactions (note that there also are some crucial contributions by the β1/β2 loop of Grp1-PH). The extended β1/β2 loop of Btk-PH thus plays a role in $IP_4$ binding analogous to that of the β6/β7 insertion in Grp1-PH. An intriguing issue is why two PH domains that effectively bind the same ligand with similar characteristics should interact with the 5-phosphate by such distinct mechanisms. One obvious possibility is that

**Figure 1**




Comparison of the **(a)** DAPP1, **(b)** Grp1 and **(c)** Btk-PH structures. Elements of secondary structure are coloured: blue for α-helices and green for β-strands. The seven β-strands of the PH domain core are labelled 1-7, and both amino and carboxy termini are marked. The phosphate positions of the bound $IP_4$ are also shown. The two strands present in the insertion between β6 and β7 of Grp1 (β6′ and β6″) are coloured red, as is the extended β1/β2 loop of Btk.

the β6/β7 insertion has another function besides supplying residues for $IP_4$ binding.

Without doubt, these new crystal structures [4–6] have enhanced our understanding of how certain PH domains bind 3-phosphoinositides, but can we predict the specificity of an as yet uncharacterised PH domain solely from the primary sequence? Previous work identified a motif of three basic residues, located in the β1 and β2 strands, that are strictly conserved in PH domains known to bind 3-phosphoinositides [7]. Lietzke *et al.* [6] have now extended this motif to a distinctive 'signature motif' for 3-phosphoinositide binding: [LVIMF]-X-K-[GASP]-$X_m$-[WFA]-(KR)-X-R-X-[FL]-X-[LM]-$X_n$-[LIF]-X-Y, where X denotes any residue and n and m are integers in the ranges 5–10 and 6–13, respectively.

On the basis of the structural considerations described briefly above, Ferguson *et al.* [5] elegantly predicted that, if a 3-phosphoinositide-binding PH domain has a short β1/β2 loop with no other insertion to substitute for it, and has no basic residue at the beginning of the β1/β2 loop, then it should not be capable of forming a binding pocket for the 5-phosphate. Such a PH domain is thus likely to bind similarly well both $PIP_3$ and $PI(3,4)P_2$. Using these predictions, they identified a PH domain from a sequence database (accession number AA054961), and demonstrated that it does indeed bind both $PI(3,4)P_2$ and $PIP_3$. Thus in this case, at least, the phosphoinositide-binding specificity of a PH domain has been successfully predicted from the primary sequence. Whether this can be extrapolated to $PIP_3$-specific PH domains, given the distinct mechanisms for interaction with the 5-phosphate, is still a matter of some debate.

This whole issue has been made potentially more difficult by the remarkable demonstration [8] that addition of a single glycine residue to Grp1-PH can have a significant effect on its polyphosphoinositide-binding specificity. It is a somewhat puzzling fact that two splice variants of Grp1 are made [9]. Referred to as the diglycine and triglycine forms, these variants differ only by the presence or absence of a third glycine residue in the β1/β2 loop. The genes for other members of the cytohesin family — ARNO, cytohesin-1 and cytohesin-4 — encode a similar alternative exon to that conferring the glycine difference in Grp1. From quantitative RT-PCR, the percentage of the diglycine form of a cytohesin made can vary from 10%–90%, depending on the family member and the cell type [9].

Interestingly, whereas the diglycine form of Grp1-PH has dissociation equilibrium constants ($K_d$s) for $PIP_3$ and phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) of 0.2 and 170 μM, respectively, in the triglycine form the corresponding values are 0.6 and 20 μM [8]. The triglycine form of the protein thus shows much less discrimination between $PI(4,5)P_2$ and $PIP_3$ than the diglycine form. From the Grp1-PH structures [5,6] — both generated with the diglycine form — it appears that the reduction in affinity for $PIP_3$ probably results from an alteration in the conformation of the β1/β2 loop, reducing some of the interactions with the 5-phosphate. How the extra glycine causes an increase in the $PI(4,5)P_2$ affinity is more difficult to comprehend. One possibility is that the third glycine 'opens

up' the binding pocket, allowing the $PI(4,5)P_2$ to enter in an orientation — possibly more akin to the $PI(4,5)P_2$ binding site of $PLC\delta_1$-PH [10] — that is sterically hindered in the diglycine form. Only solving the structure of ligand-bound triglycine Grp1-PH will resolve this issue.

The really important issue, however, is why do cells produce two forms of Grp1 with different inositol lipid specificities? Although there is no obvious reason for this in Grp1, the same cannot be said for the Grp1-related protein ARNO. In this instance, whereas the diglycine ARNO-PH has $K_d$s for $PIP_3$ and $PI(4,5)P_2$ of 2 and 117 μM, the corresponding $K_d$s in the triglycine form are 1 and 3 μM, respectively (curiously there is no real difference between the $PIP_3$ affinities of these two forms of ARNO). Thus, although a 34-fold selectivity for $PIP_3$ versus $PI(4,5)P_2$ is retained in triglycine Grp1-PH, this is reduced to only a two-fold selectivity in triglycine ARNO-PH. Furthermore, the affinity for binding $PI(4,5)P_2$ in the latter case is significantly higher that of the diglycine form of the protein, an observation that may have an important baring on the membrane association of triglycine ARNO.

As highlighted by Lemmon and Ferguson [3], for a PH domain to drive PI 3-kinase-dependent plasma membrane recruitment, the following criteria must be fulfilled. First, the selectivity for $PIP_3$ versus $PI(4,5)P_2$ must be greater than about 25. Secondly, the affinity for $PI(4,5)P_2$ must be lower than about 10 μM, as the constitutively plasma membrane localised $PLC\delta_1$-PH binds $PI(4,5)P_2$ with a $K_d$ of 2 μM, whereas the non-membrane associated PH domain from pleckstrin binds $PI(4,5)P_2$ with a $K_d$ of 30 μM. Finally, the $K_d$ for $PIP_3$ must be less than about 1 μM. Clearly, whereas both Grp1-PH domains and the diglycine ARNO-PH fulfill these criteria, the triglycine ARNO-PH does not. Taken together with the fact that the triglycine ARNO-PH has an affinity for $PI(4,5)P_2$ almost identical to $PLC\delta_1$-PH, it seems likely that, as with the $PLC\delta_1$-PH, triglycine ARNO-PH may be constitutively associated with the plasma membrane (note, this argument is based on affinities for the isolated ARNO-PH domains and not the entire protein [8]).

Such a conclusion may help to explain the following apparent contradiction. In studies using overexpression of tagged constructs, ARNO has been described as either a cytosolic protein that undergoes PI 3-kinase-dependent plasma membrane recruitment [11], or a protein of which a significant proportion is constitutively membrane associated [12]. It now appears that, whereas the former study used the diglycine form of ARNO, the latter work was performed with the triglycine version. Importantly, as the ability of ARNO to activate members of the ARF family of small G proteins is regulated by membrane association [11,12], constitutive versus $PIP_3$-driven membrane targeting may give rise to distinct profiles for the activation of downstream ARF signalling. Thus, although we are learning a lot about PH domains that interact with inositol lipids, the added complexity provided by the diglycine versus triglycine forms suggests that we are far from fully understanding the subtleties of this crucial signalling domain.

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